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Patents ADP number *(if you know it)*	6254007002	
If the applicant is a corporate body, give the country/state of its incorporation	UNITED KINGDOM	
4. Title of the invention	PROTEIN COMBINATIONS	
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PROTEIN COMBINATIONS

The present invention relates to *inter alia*, an insecticidal synergistic combination of proteins, DNA sequences encoding the proteins and methods of producing plants comprising the said combination. The present invention also provides peptides and DNA sequences encoding them, processes for their manufacture and use, and transgenic plants transformed with constructs encoding said peptides. In particular the invention relates to insecticidal peptides isolatable from the genus *Paecilomyces.*

Many fungi are pathogenic to insects. It is known that *Peacilomyces fumosoroseus* can be used as a biological control agents and this strain is sold commercially as a biocontrol agent for use in greenhouses. No work has, however, been carried out looking for gene-encoded insecticidal peptides from genus *Paecilomyces.*

The applicants have found that insecticidal peptides can be extracted from of *Paecilomyces spp* and have now purified a new type of potent orally active insecticidal peptide from strains of *Paecilomyces spp.* in particular *Paecilomyces farinosus*. We have also found that when these proteins are capable of acting synergistically with further proteins.

According to the present invention there is provided an insecticidal synergistic combination comprising a first protein depicted as SEQ ID No 43 and at least one further protein. Preferably, the said first protein comprises the sequence depicted as SEQ ID No 1 and the said further protein is an insecticidal CRY protein. The term "CRY protein" includes crystal endotoxin proteins and the vegetative insecticidal proteins (secreted VIPs) which are active against insects including Lepidoptera, Coleoptera and Diptera. Such proteins are available *inter alia*, from the bacterium *Bacillus thuringienesis* and are well known to the person skilled in the art. Particularly preferred CRY proteins which may be used in accordance with the present invention include those proteins obtainable from *Bacillus thuringienesis* variety tenebrionis which has been deposited under the German Collection of micro-organisms (Deutsche Sammlung von Microorganism) under reference DSM 2803 or strains JHCC 4835 and JHCC 4353 deposited under the National Collections of Industrial and Marine Bacteria (Aberdeen) under the accession numbers NCIMB 40091 and 40090, respectively. Further preferred are the proteins depicted as SEQ ID Nos 44 to

49. More preferably the said further protein is selected from the group of sequences depicted as SEQ ID Nos. 44 to 49.

The present invention further provides a polynucleotide which comprises a region encoding the said first and further protein as described above.

The present invention further provides a method of providing a plant or plant part with a combination described above comprising: (a) inserting into a plant cell a polynucleotide as described above; and (b) regenerating a plant from said cell; and (c) selecting the plant or plant part having the said combination.

The present invention further provides a method of providing a plant or plant part with a combination as described above comprising: (a) inserting into a plant cell which is capable of producing a further protein, a polynucleotide which provides for the production of a first protein depicted as SEQ ID No 43; and (b) regenerating a plant from the said cell; and (c) selecting the plant or plant part having the said combination or, (a) inserting into a plant cell which is capable of producing a first protein depicted as SEQ ID No 43, a polynucleotide which provides for a further protein; and (b) regenerating a plant from the said cell; and (c) selecting the plant or plant part having the said combination. The present invention further provides a method of providing a plant with a combination as described above comprising crossing a first plant which is capable of producing a first protein depicted as SEQ ID No 43 with a second plant which is capable of producing a further protein and selecting the resultant plant which is capable of producing the said combination. In the methods of the preceding paragraph it is preferred that the said first protein has the sequence depicted as SEQ ID No 1 and the said further protein is an insecticidal CRY protein.

The present invention further provides any plant obtained by the methods described above.

The present invention further provides a plant cell which comprises an insecticidal synergistic combination as described above and a plant comprising such cells.

The present invention still further provides a method of controlling insects comprising providing at a locus where the said insects feed, a combination as described above.

In a further aspect of the present invention there is provided a method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising: (a)

providing a population of variants of said polynucleotide and further polynucleotides which encode further proteins, at least one of which is in cell free form; and (b) shuffling said variants and further polynucleotides to form recombinant polynucleotides; and (c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and (d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least a polynucleotide encoding the protein depicted as SEQ ID No. 43. It is preferred that the said population of variants in part (a) contains at least a polynucleotide encoding the protein depicted as SEQ ID No.43 and at least one of the said further polynucleotides encodes a CRY protein.

The present invention further provides a method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising: (a) providing a population of variants of said polynucleotide and further polynucleotides which encode further proteins, at least one of which is in cell free form; and (b) shuffling said variants and further polynucleotides to form recombinant polynucleotides; and (c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and

(d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least a polynucleotide which comprises a region encoding the said first and further protein as described above.

The present invention further provides a method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising: (a) providing a population of variants of said polynucleotide, at least one of which is in cell free form; and (b) shuffling said variants to form recombinant polynucleotides; and (c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and (d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least

a polynucleotide encoding the protein depicted as SEQ ID No. 43. Preferably, the said population of variants in part (a) of the preceding sentence contains at least a polynucleotide encoding the protein depicted as SEQ ID No. 1.

The present invention further provides a polynucleotide obtainable or obtained by the methods described above and a protein encoded by the said polynucleotide.

The present invention further provides the use of an evolved polynucleotide obtainable or obtained by the methods described above in the production of plants or plant parts which are resistant to infestation by insects and the use of such an evolved polynucleotide in the production of a protein which is capable of producing a synergistic combination with a further protein.

According to the present invention there is further provided a polynucleotide encoding a fusion protein comprising a first protein depicted as SEQ ID No 43 and at least one further protein. Preferably the said first protein has the sequence depicted as SEQ ID No. 1 and the said further protein is a CRY protein preferably selected from the group consisting of SEQ ID Nos. 44 to 49. The polynucleotide encoding the said fusion protein preferably further comprises a sequence encoding a region which separates the said first and further proteins within the said fusion protein.

The present invention further provides a fusion protein encoded by a polynucleotide according to the preceding paragraph.

The present invention further provides a method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising: (a) providing a population of variants of said polynucleotide, at least one of which is in cell free form; and (b) shuffling said variants to form recombinant polynucleotides; and (c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and (d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least a polynucleotide encoding a fusion protein as described above. The methods for evolving a polynucleotide as described above are well known to the person skilled in the art and are described *inter alia*, in US Patent No. 5,811,238.

The present invention further provides a plant comprising a polynucleotide described above.

The present invention provides a peptide obtainable from the genus of *Paecilomyces,* and having insecticidal properties or a fragment thereof, or a homologue, variant or derivative of any of these which has insecticidal activity. Suitably the peptide is isolated in the sense that it is substantially free from other proteins and peptides with which it is associated in *Paecilomyces*. Specifically, the protein in substantially pure form.

The term "peptide" used herein encompasses both small peptides and large polypeptides including proteins.

Once isolated from *Paecilomyces*, an insecticidal peptide can be characterised using conventional methods, and then fragments, variants of homologues having insecticidal activity can be derived. Suitably the peptide is an insecticidal peptide obtainable from the genus *Paecilomyces,* such as *Paecilomyces farinosus.*

In particular, the invention provides an insecticidal peptide comprising the amino acid sequence

GKICTPAGVKCPAALPCCPGLRCIGGVNNKVCR (SEQ ID NO 1)

or a fragment thereof, or a homologue, variant or derivative of any of these which has insecticidal activity.

SEQ ID NO 1 is a peptide derivable from *Paecilomyces farinosus.*

As used herein the expression "fragment" refers to any portion of the given amino acid sequence which has insecticidal activity either alone or when combined with other portions of the amino acid sequence. Fragments will suitably comprise at least 5, and preferably at least 10 consecutive amino acids from the basic sequence.

The expression "homologues" as used herein refers to any peptide which has some amino acids in common with the given sequence. Suitably at least 60% of the amino acids will be similar, more suitably at least 70%, preferably at least 80%, more preferably at least 90% and most preferably at least 95% of amino acids will be similar to the corresponding amino acid in the given sequence.

As used herein the term "similar" is used to denote sequences which when aligned have similar (identical or conservatively replaced) amino acids in like positions or regions, where identical or conservatively replaced amino acids are those which do not alter the activity or function of the protein as compared to the starting protein. For example, two amino acid sequences with at least 85% similarity to each other have at least 85% similar (identical or conservatively replaced) amino acid residues in a like position when aligned optimally allowing for up to 3 gaps, with the *proviso* that in respect of the gaps a total of not more than 15 amino acid resides is affected. The degree of similarity may be determined using methods well known in the art (see, for example, Wilbur, W.J. and Lipman, D.J. "Rapid Similarity Searches of Nucleic Acid and Protein Data Banks." Proceedings of the National Academy of Sciences USA 80, 726-730 (1983) and Myers E.and Miller W. "Optimal Alignments in Linear Space". Comput. Appl. Biosci. 4:11-17(1988)). One programme which may be used in determining the degree of similarity is the MegAlign Lipman-Pearson one pair method (using default parameters) which can be obtained from DNAstar Inc, 1228, Selfpark Street, Madison, Wisconsin, 53715, USA as part of the Lasergene system.

Amino acids which differ from the basic sequence may be conservatively or non-conservatively substituted. A conservative substitution is to be understood to mean that the amino acid is replaced with an amino acid with broadly similar chemical properties. In particular conservative substitutions may be made between amino acids with the following groups:

(i) Alanine, Serine, Glycine and Threonine;

(ii) Glutamic acid and Aspartic acid;

(iii) Arginine and Lysine;

(iv) Asparagine and Glutamine;

(v) Isoleucine, Leucine, Valine and Methionine;

(vi) Phenylalanine, Tyrosine and Tryptophan.

In general, more conservative than non-conservative substitutions will be possible without destroying the insecticidal properties of the compounds. Suitable homologues may be determined by testing insecticidal properties of the peptide using routine methods, for example as illustrated hereinafter.

The term "variant" as used herein includes experimentally generated variants or members of a family of related naturally-occurring peptides as may be identified by molecular genetic techniques. Such techniques are described for example in US Patent No. 5,605,793, US Patent No. 5,811,238 and US Patent No 5,830,721, the content of which is incorporated herein by reference. In essence this technique involves expression of the parental gene in a microbial expression system such as *Escherichia coli*. The particular system selected must be validated and calibrated to ensure that biologically active peptides are expressed, which may be readily achieved using a *in vivo* bioassay. The gene, or preferably a collection of related genes from different species, may be subject to mutagenic polymerase chain reaction (PCR) as is known in the art. Fragmentation of the products and subsequent repair using PCR leads to a series of chimeric genes reconstructed from parental variants. These chimeras are then expressed in the microbial system which can be screened in the usual way to determine active mutants, which may then be isolated and sequenced. Reiteration of this molecular evolution DNA shuffling cycle may lead to progressive enhancement of the desired gene properties. The advantage of a technique of this nature is that it allows a wide range of different mutations, including multi-mutation block exchanges, to be produced and screened.

Particular variants are those derivable from peptides of SEQ ID NO 1 or homologues thereof, in particular those isolatable from *Paecilomyces spp.* . Other particular variants are those which are experimentally generated using for example the molecular evolution techniques. Preferably such variants will have improved insecticidal activity or function as compared to the native sequences. Suitable improvements may be in relation to the intrinsic specific activity of the protein, the specificity or target range against which the peptide is active or by altering a physical property such as stability.

In a further aspect, the invention provides the use of a peptide or peptides comprising SEQ ID NO 1, in the production of other insecticidal variants using molecular evolution and/or DNA shuffling methods.

Other variants may be identified or defined using bioinformatics systems. An example of such a system is the FASTA method of W.R. Pearson and D.J. Lipman PNAS (1988) 85:2444-2488. This method provides a rapid and easy method for comparing protein sequences and detecting levels of similarity and is a standard tool, used by molecular

biologists. Such similar sequences may be obtained from natural sources, through molecular evolution or by synthetic methods and comparisons made using this method to arrive at "opt scores" which are indicative of the level of similarity between the proteins.

Particular variants of the invention will comprise insecticidal peptides with an amino acid sequence with a FASTA opt score (as defined in accordance with FASTA version 3.0t82 November 1, 1997) against SEQ ID NO 1 of greater than 109, for example in excess of 120, more preferably in excess of 150 and most preferably in excess of 190.

Variants which give FASTA scores in excess of 199 when compared with SEQ ID NO 1 are particularly preferred.

With these constraints in mind, a skilled person would be able to isolate other members of the family of peptides, for example by designing probes or primers based upon SEQ ID NO 1 but modified within the limits of the FASTA opt score range. These probes could then be used to screen libraries such as cDNA or genomic libraries using conventional methods, in particular those derived from fungal species, in order to isolate other family members. Hybridisation conditions used during these screening exercises are either low or high stringency, preferably high stringency conditions as are routinely used in the art (see for example "Molecular Cloning, A Laboratory Manual" by Sanbrook et al, Cold Spring Harbor Laboratory Press, N.Y.). In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three times as strong as 1x SSC and so on.

Once found other family members could also be subject to molecular evolution techniques or DNA shuffling as described herein, in order to improve the properties thereof. All peptides obtained in this way should be regarded as a variant within the ambit of the present invention.

The term "derivative" relates to peptides which have been modified for example by using known chemical or biological methods. This includes acetylated or glycosylated forms of the peptides. It is believed that in particular the glycine at position 1 of the naturally occurring version of SEQ ID NO 1 is acetylated.

In particular, in this case, the insecticidal peptides of SEQ ID NO 1 contains 6 cysteine residues all of which are believed to be involved in forming 3 intramolecular

disulphide bonds. Thus the arrangement of the cysteine residues may be important in conferring insecticidal activity on the peptide. Therefore, homologues or variants suitably retain the cysteine residues of the combination of SEQ ID NO 1.

Thus the invention encompasses peptides which may be represented as $-AA_1-AA_2-AA_3-Cys_4-AA_5-AA_6-AA_7-AA_8-AA_9-AA_{10}-Cys_{11}-AA_{12}-AA_{13}-AA_{14}-AA_{15}-AA_{16}-Cys_{17}-Cys_{18}-AA_{19}-AA_{20}-AA_{21}-AA_{22}-Cys_{23}-AA_{24}-AA_{25}-AA_{26}-AA_{27}-AA_{28}-AA_{29}-AA_{30}-AA_{31}-Cys_{32}-AA_{33}$ (SEQ ID No 43) or a fragment thereof, with the *proviso* that the amino acids depicted at positions AA1 -3, 5-10, 12-16, 19-22, 24-31 and 33 refer to any amino acid other than cysteine. In this formula, at least some and preferably a substantial portion AA1 -3, 5-10, 12-16, 19-22, 24-31 and 33 will be the same as in the corresponding amino acids in SEQ ID NO 1 above. In particular, it would be expected that at least 85% of the amino acids corresponding to AA1 -3, 5-10, 12-16, 19-22, 24-31 and 33 of SEQ ID NO 1, are either identical or conservatively substituted as defined above.

Peptides of the invention may be used alone or they may be fused to other peptides or proteins so as to form chimeric peptides or proteins. Suitably, the other peptide or protein of the chimera will have some insecticidal effect of its own, or will act as a targeting sequence to target the insecticidal peptide to a particular site in the target pest.

The above described peptides may be prepared in various ways. For example, they may be extracted and purified from *Paecilomyces* isolates. However, since the peptide sequence is known, it may be more convenient to manufacture the peptides, either by chemical synthesis using a standard peptide synthesiser, or using recombinant DNA technology to express the peptide or variants of that peptide in suitable host cells. Suitable cells include prokaryotic or eukaryotic organisms, in particular micro-organisms such as *E. coli, Saccharomyces cerevisiae* or *Pichia pastoris*. Nucleic acids encoding the peptides, as well as vectors, host cells and methods of producing the peptides form further aspects of the invention.

In particular, the invention further provides a nucleic acid which encodes an insecticidal peptide as described above. The nucleic acid sequence may be a DNA or RNA sequence. In particular the DNA may be a cDNA sequence or a genomic sequence, and may be derived from a cDNA clone, a genomic DNA clone or DNA manufactured using a

standard nucleic acid synthesiser. A particular nucleic acid sequence which encodes a peptide of SEQ ID NO 1 is the nucleic acid of SEQ ID NO 9 as shown in Figure 3.

The DNA sequence may be predicted from the known amino acid sequence and DNA encoding the peptide may be manufactured using a standard nucleic acid synthesiser. Alternatively, the DNA sequence may be isolated from fungal-derived DNA libraries. Suitable oligonucleotide probes may be derived from the known amino acid sequence and used to screen a cDNA library for cDNA clones encoding some or all of the peptide.

The sequence of the gene encoding the protein of SEQ ID NO 1 has been obtained (SEQ ID NO 2) and is shown in Figure 2 hereinafter. The sequence was determined using reverse transcription of RNA from these fungi and Rapid Amplification of cDNA Ends (RACE) by Polymerase Chain Reaction (PCR). The 3' region of the cDNA was isolated first, using the N-terminal amino acid sequence of the mature peptide for degenerate primer design. The specific sequence obtained from the 3'RACE fragment was then used to design specific primers for amplification of the 5' region of the corresponding cDNA. Similar techniques could be employed for any other members of the same gene family. A schematic diagram of the gene sequence is shown in Figure 1. As well as a signal sequence of 17 amino acids, and a coding sequence for the peptide of SEQ ID NO 1 followed by a TAA stop codon, the gene has a 5' untranslated region (5'UTR) of approximately 110 base pairs and a 3'untranslated region (3'UTR) of approximately 160 base pairs.

Using the natural coding sequence SEQ ID NO 2 or fragments thereof such as SEQ ID NO 9, naturally occurring variants can be isolated. These will include DNA which hybridises to the naturally occurring sequence or fragments thereof. Preferably, such hybridisation occurs at, or between, low and high stringency conditions, which have been defined above.

Particular homologues of SEQ ID NO 1 are homologues identified in other insecticidal fungi either by protein purification and sequence analysis or by amplification from fungal genomes and/or cDNA preparations using PCR primers based on SEQ ID NO 1.

The peptide of SEQ ID NO 1 is a secreted peptide and therefore DNA encoding it which is isolated from *Paecilomyces spp* contains a signal sequence in addition to the DNA sequence encoding the mature peptide. Figure 2 illustrates the signal sequence in bold type,

whilst and the sequence of the mature peptide is underlined. The signal sequences (SEQ ID NOS 7 and 8 below) form a further aspect of the invention.

Oligonucleotide probes or cDNA clones may be used to isolate the gene or genes which encode the insecticidal peptide by screening genomic DNA libraries.

The DNA sequence encoding the insecticidal peptide may be incorporated into a DNA construct or vector in combination with suitable regulatory sequences (promoter, terminator, etc). Genes encoding the peptides of the invention can be expressed in a variety of systems, including monocotyledonous plants, dicotyledenous plants and microbial systems as would be known in the art. The components of the constructs such as the promoter, terminator, selectable marker can be chosen to ensure that the peptide is expressed well in the particular expression system. Examples are illustrated hereinafter. For example, the DNA sequence may be placed under the control of a constitutive or an inducible promoter (stimulated by, for example, environmental conditions, presence of a pest, presence of a chemical). Such a DNA construct may be cloned or transformed into a biological system which allows expression of the encoded peptide. Suitable biological systems include micro-organisms (for example, bacteria such as *Escherichia coli*, *Pseudomonas* and endophytes such as *Clavibacter xyli* subsp. *cynodontis* (Cxc); yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris*; viruses; bacteriophages; etc), cultured cells (such as insect cells, mammalian cells) and plants. The expressed peptide may be isolated and if necessary formulated, for use. Alternatively, the peptide may be expressed *in situ* or *in vivo* under circumstances where they will be directly brought into contact with the target pests.

It has been found that the peptides of the invention are insecticidal whether applied to pests either orally or by injection. Pests affected in this way include lepidopteran pests, for example as illustrated hereinafter and dipteran species such as the fruit fly *Drosophila melanogaster*. Thus the invention further provides a method of killing or controlling insect pests which comprises administering to said pests or to the environment thereof, a peptide as described above.

For agricultural applications, the insecticidal peptide may be used to improve the pest insect-resistance or pest insect-tolerance of crops either during the life of the plant or for post-harvest crop protection. Pests exposed to the peptides are inhibited. The insecticidal

peptide may eradicate a pest already established on the plant or may protect the plant from future pest attack.

Exposure of an insect pest to an insecticidal peptide of the invention may be achieved in various ways, for example:

(a) a composition comprising peptide may be applied to the insect or to the environment in which they live, in particular, to plant parts or the surrounding soil, using standard agricultural techniques (such as spraying);

(b) a composition comprising a micro-organism such as an insect virus, genetically modified to express the insecticidal peptide may be applied to a plant or the soil in which a plant grows;

(c) an endophyte genetically modified to express the insecticidal peptide may be introduced into the plant tissue (for example, via a seed treatment process);

An endophyte is defined as a micro-organism having the ability to enter into non-pathogenic endosymbiotic relationships with a plant host. A method of endophyte-enhanced protection of plants has been described in a series of patent applications by Crop Genetics International Corporation (for example, International Application Publication Number WO90/13224, European Patent Publication Number EP-125468-B1, International Application Publication Number WO91/10363, International Application Publication Number WO87/03303). The endophyte may be genetically modified to produce agricultural chemicals. International Patent Application Publication Number WO94/16076 (ZENECA Limited) describes the use of endophytes which have been genetically modified to express a plant-derived insecticidal peptide.

(d) DNA encoding an insecticidal peptide may be introduced into the plant genome so that the peptide is expressed within the plant body (the DNA may be cDNA, genomic DNA or DNA manufactured using a standard nucleic acid synthesiser).

Where method (a) or (b) above is used, the peptide or micro-organism is generally applied in the form of an insecticidal composition. Such compositions, which form a further aspect of the invention, will generally further comprise an agriculturally acceptable carrier or diluent as is known in the art. Suitable carriers or diluents are solids or liquids. Concentrates in the form of solids or liquids may be prepared, which require dilution in water prior to application, for example by spraying.

Preferably, the peptides of the invention are administered in accordance with method (d) above. Thus in a preferred embodiment, nucleic acids of the invention utilise codons which are particularly preferred in plants.

Examples of preferred codon usage from cotton and maize plants is set out in Table 1

Table 1

Amino Acid	Cotton preference	Maize preference
Alanine	GCT	GCC
Arginine	AGG	AGG
Asparagine	AAC	ACC
Aspartic Acid	GAT	GAC
Cysteine	TGC	TGC
Glutamine	CAA	CAG
Glutamic Acid	GAG	GAG
Glycine	GGT	GGC
Histidine	CAT	CAC
Isoleucine	ATT	ATC
Leucine	CTT	CTG
Lysine	AAG	AAG
Methionine	ATG	ATG
Phenylalanine	TTC	TTC
Proline	CCT	CCG
Serine	TCT	AGC
Threonine	ACT	ACC
Tryptophan	TGG	TGG
Tyrosine	TAC	TAC
Valine	GTT	GTG

The table shows that the codon usage for these two species is fairly similar, and even those preferred codons which differ are fairly compatible (e.g. they are the second most preferred codon). Thus a codon optimised DNA coding sequence for maize and cotton in respect of SEQ ID NO 1 can be readily generated.

Plant cells may be transformed with recombinant DNA constructs according to a variety of known methods (*Agrobacterium* Ti plasmids, electroporation, microinjection, microprojectile gun, etc). The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocotyledonous and dicotyledonous plants may be obtained in this way, although the latter are usually more easy to regenerate. Some of the progeny of these primary transformants will inherit the recombinant DNA encoding the insecticidal peptide(s).

Thus the invention further provides a plant containing recombinant DNA which expresses an insecticidal peptide according to the invention. Such a plant may be used as a parent in standard plant breeding crosses to develop hybrids and lines having improved insect resistance.

Suitably the recombinant DNA is incorporated such that it is expressed in a region of the plant which is subject to pest attack (such as the leaves) and is therefore ingested by the pest. The DNA may comprise sequences which enhance or control this or which are necessary for the mature peptide to fold correctly. For example, the nucleotide sequence encoding the peptide may be under the control of a promoter which is expressed particularly in the desired tissues. Other methods of targeting the peptide are possible. For example, the nucleic acid may further comprise a signal sequence which targets the peptide to the apoplast (extra-cellular space) as a general expression location in the plant. Suitable signal sequences include those derived for example from the Dahlia antifungal peptide Dm-AMP-1 and the Radish antifungal peptide Rs-AFP1, the Maize hydroxyproline-rich glycoproten (HRGP) signal peptide and the Tobacco PR-1a signal sequence which are as follows:

Dahlia:

SEQ ID NO 4: -Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val
SEQ ID NO 3:- ATG GTT AAT AGA TCT GTT GCT TTT TCT GCT TTT GTT
Leu Ile Leu Phe Val Leu Ala Ile Ser Asp Ile Ala
CTT ATT CTT TTT GTT TTG GCT ATT TCA GAT ATT GCT
Ser Val Ser Gly
TCT GTT TCA GGA

Radish:

SEQ ID NO 6:-Met Ala Lys Phe Ala Ser Ile Ile Ala Leu Leu Phe

SEQ ID NO 5:-ATG GCT AAG TTT GCT TCT ATT ATT GCT CTT TTG TTT

Ala Ala Leu Val Leu Phe Ala Ala Phe Glu Ala Pro

GCT GCA CTT GTT TTG TTT GCT GCA TTT GAA GCT CCA

Thr Met Val Glu Ala

ACT ATG GTT GAA GCT

Maize HRGP signal sequence:

SEQ ID NO 11:-Met Gly Gly Ser Gly Lys Ala Ala Leu Leu Leu Ala Leu

SEQ ID NO 10:-ATG GGT GGC AGC GGC AGG GCT GCT CTG CTG CTG GCC CTG

Val Val Val Ala Val Ser Leu Ala Val Glu Ile Gln Ala

GTG GTG GTG GCC GTG AGC CTG GCC GTG GAG ATC CAG GCC

Tobacco PR-1a signal sequence

SEQ ID NO 13:- Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu

SEQ ID NO 12 ATG GGA TTT GTT CTC TTT TCA CAA TTG CCT TCA TTT CTT

Leu Val Ser Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg Ala

CTT GTC TCT ACA CTT CTC TTA TTC CTA GTA ATA TCC CAC TCT TGC CGT

GCC

A further suitable signal sequence is the signal sequence of the gene encoding SEQ ID NO 1 from *Paecilomyces farinosus* which can be represented as follows:

SEQ ID NO 8:-Met Gln Ile Ser Ala Val Ile Val Ala Leu Phe Ala

SEQ ID NO 7:-ATG CAA ATC TCC GCC GTC ATT GTC GCA CTC TTC GCC

Ser Ala Ala Met Ala

AGC GCC GCC ATG GCC

These or other signal sequences can be used as pre-protein signals and the sequence encoding the insecticidal peptide of the invention may then be placed at the C-terminal end of the chimeric protein.

Transgenic plants in accordance with the invention show improved resistance or enhanced tolerance to an insect pest when compared to a wild-type plant. Resistance may vary from a slight increase in tolerance to the effects of the pest (where the pest is partially inhibited) to total resistance so that the plant is unaffected by the presence of pest (where the pest is severely inhibited or killed). An increased level of resistance against a particular pest or resistance against a wider spectrum of pests may both constitute an improvement in resistance. Transgenic plants (or plants derived therefrom) showing improved resistance are selected following plant transformation or subsequent crossing.

Examples of genetically modified plants which may be produced include field crops, cereals, fruits and vegetables such as canola, sunflower, tobacco, barley, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, cabbage, onion, etc. Particularly preferred genetically modified plants are sugar beet, cotton, maize, wheat, rice, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax and oilseed rape, and nut producing plants.

As the insecticidal peptides of the invention are very active against some of the major cotton pests, it would be particularly advantageous to transform cotton plants with constructs encoding said peptides. Alternatively, the peptides may be supplied to cotton plants by any other suitable method.

The invention still further includes the progeny of the plants of the preceding paragraph, which progeny comprises the said polynucleotide, or functionally sufficient parts thereof, stably incorporated into its genome and heritable in a Mendelian manner and the seeds of such plants and such progeny.

Plant transformation, selection and regeneration techniques, which may require routine modification in respect of a particular plant species, are well known to the skilled man.

The insecticidal peptides of the invention may be employed alone or in combination with other agrochemicals such as herbicides, fungicides or, most suitably, other insecticidal compounds such as insecticidal peptides and proteins. Thus insecticidal compositions in

accordance with the invention may comprise additional agrochemical compounds. Where the other compounds are peptides or proteins, nucleic acids encoding these may be included in the composition in the form of expression vectors. Where these are used, the additional nucleic acids may be in the same vector as the peptide of the invention, or in additional vectors.

Examples of possible mixture partners include insecticidal lectins, insecticidal protease inhibitors and insectidal proteins derived from species of the *Bacillus thurigiensis* , *Xenorhadus nematophilus*, or *Photorabdus luminescens*.

The invention will now be described by way of example only with reference to the drawings, in which:

Figure 1 is a schematic diagram of the organisation of the *Paecilomyces farinosus* gene.

Figure 2 (SEQ ID No. 2) shows the nucleotide sequence of the natural gene encoding SEQ ID NO 1 as well as the amino acid sequence, with the signal sequence shown in bold type and the sequence of the mature peptide underlined.

Figure 3 shows SEQ ID NO 9 which is the natural coding sequence of the mature peptide of SEQ ID NO 1.

Figure 4 shows diagramatically a construct suitable for the transformation of corn where A illustrates the signal-gene fusion part of the construct and B illustrates a backbone vector.

Figure 5 shows SEQ ID No. 43.

Figure 6 shows SEQ ID No. 44 cry1Ia1 (Embl. Accession No. X62821).

Figure 7 shows SEQ ID No. 45 cry1Ia2 (Embl. Accession No. M98544).

Figure 8 shows SEQ ID No. 46 cry1Ia3 (Embl. Accession No. L36338).

Figure 9 shows SEQ ID No. 47 cry1Ia4 (Embl. Accession No. L49391).

Figure 10 shows SEQ ID No. 48 cry1Ia5 (Embl. Accession No. Y08920).

Figure 11 shows SEQ ID No. 50 cry1Ib1 (Embl. Accession No. U07642).

Example 1

Culturing of *Paecilomyces farinosus*

Paecilomyces farinosus was routinely cultured on potato dextrose agar plates. Spores were harvested from the plates by adding sterile water and scraping with a sterile spatula. For production of insecticidal peptide $6x10^7$ spores were inoculated into 5x 200ml of SDB

medium in 500ml flasks. Cultures were incubated at 24°C with shaking at 180rpm for 7 days before harvest.

Example 2

Purification of insecticidal peptide

500ml of 7d culture filtrate was filtered through Whatman GF/B paper to remove mycelium and the supernatant diluted 4 fold in 20mM MES pH6. The supernatant was then loaded onto a S-Sepharose FF XK16/10 column (Pharmacia Biotech) previously equilibrated with 20mM MES pH6. Unbound protein was washed through the column with 3 column volumes of 20mM MES pH6 and bound protein was eluted with a linear gradient of 0-1M NaCl in 20mM MES pH6 over 20 column volumes. The eluate was monitored for peptide by online measurement of absorbance at 280 and 210nm. 5ml fractions were collected and following dialysis against 50mM Sodium Phosphate buffer pH7 assayed against *Heliothis virescens*.

Active fractions eluted around 250mM NaCl. These fractions were pooled and following concentration on Polyethylene glycol Mwt 20,000, were further purified by reverse phase. 2ml of sample was loaded onto a 3ml Resource RPC column (Pharmacia Biotech) and bound peptide eluted with a linear gradient of 0.05% trifluoroacetic acid (TFA) to 50% acetonitrile, 0.0% TFA over 20 column volumes. The eluate was monitored for absorbance at 210 and 280nm. The active peak eluted at approximately 20% acetonitrile.

Example 3

Identification of peptide sequence

The sequence of the active peptide in the product of Example 2 could not be determined directly, probably due to a blocked N-terminus. The peptide was reduced and subjected to and tryptic digestion. This yielded a series of fragments which could be sequenced using Edman degradation methods. Using a combination of this, and mass spectrometry, the sequence of the peptide was determined as being SEQ ID NO 1. The mass spectrometry data indicates that the N-terminal glycine is acetylated.

Example 4

Biological Activity in insect bioassay

The isolated peptide was bioassayed against a range of insect species using the following method:

Prior to the assay twenty neonate lepidoptera larvae were gently brushed into each of three 'minipots' containers per treatment (i.e. three replicates per treatment). The peptide from Example 2 was diluted using 0.1% Synperonic™ solution to act as a wetter and aid the spread of the material over the waxy leaf cuticle. In spectrum assays, test materials were made up to a single high concentration, whereas in potency assays vs. *H. virescens* a rate range was tested.

Three freshly excised cotton leaves per treatment had 0.1 ml of the appropriate treatment applied by pipette to the centre of the axial surface of each leaf. The droplet was then spread over a circular area in excess of the diameter of a minipot with a fine artists paint brush (a fresh paint brush being used for each compound to avoid contamination). The leaves were left in a fume cupboard just long enough for the surface deposit to dry but care was taken to avoid excessive leaf wilting.

Once dry the leaves were placed, contaminated surface down over the appropriately labelled minipot and a lid snapped over it. The minipots were placed in plastic trays and held in a controlled temperature at 25-27°C.

After three days the numbers of live larvae remaining were counted and percent mortality determined. In the *H. virescens* potency assay the test data was run through a logit analysis package to establish the LC_{50}.

The results for 4 lepidopteran pests are shown in Table 2.

Table 2

Test Species	Rate (ppm)	% kill
Heliothis virescens	1000	100
Helicoverpa zea	1000	100
Spodoptera exigua	1000	100
Plutella xylostella	1000	100

b) Cell cytotoxicity

Two cell lines were used to determine if the peptide from Example 2 was cytotoxic to either mammalian cells (MEL cells) or insect cells (Sf21 cells). MEL cells and Sf21 cells were grown in DMEM and TC100 media respectively in 96-well microtitre plates and incubated with the appropriate concentration of peptide. The cells were scored for visible cell death after 24 hours and viability and growth assessed after 3 (MEL cells) or 4 (Sf21) days using the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to form an insoluble purple formazan as a marker for metabolically active cells.

At the highest rate tested (100 μg/ml) the peptide did not inhibit cell growth or cause any cytotoxic effects on either cell line.

Example 5

Comparison of known protein sequences to SEQ ID NO 1 using the FASTA Algorithm:

A FASTA comparative search of SEQ ID NO 1 to a database of protein sequences was carried out.

SEQ ID NO 1 was compared to all publicly available protein sequences using the FASTA method (FASTA version 3.0t82 November 1, 1997 Reference: W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448).

Specifically a large non-redundant protein database, including release 36.0 of SWISS-PROT, queried on 16 June 1999 returned proteins judged to have some similarity to SEQ ID NO 1. The best way to judge similarity using FASTA by those skilled in the art is to use the opt score output. The comparison of SEQ ID NO 1 to the non-redundant protein sequence database gave very few proteins and none with a high opt score demonstrating that SEQ ID NO 1 is not closely related to any known protein. The 'most similar' was in

GENESEQP and had an opt score of 109.

Example 6

Characterisation of natural coding sequence of peptide of SEQ ID NO 1

Harvesting of Material

A *Paecilomyces farinosus* strain having insecticidal activity was grown in Sabouraud Dextrose Broth (Difco Laboratories: 10g Bacto Neopeptone, 20g Bacto Dextrose per litre water) for 5 days at 24°C with shaking at 180 rpm.

The culture was pelleted (8000rpm, 10 minutes) and stored at -80°C until use.

RNA Extraction

Harvested material was ground to a fine powder using a pestle and mortar under liquid nitrogen. RNA was extracted from 1g of fungal pellet using the Qiagen RNeasy kit, following manufacturers specifications. The total RNA fraction was eluted from the RNeasy purification column in 1ml water.

Poly(A)+ RNA was isolated from the 700mg total RNA using the Promega PolyATtract mRNA isolation system I, following manufacturers' specifications. The Poly(A)+ RNA fraction was eluted from the magnetic beads in 1ml water, and concentrated to 15µml (of approximately 0.5mg/ml) by ethanol precipitation.

RNA samples were stored at -80°C until use.

In the following reactions, the primers and probes used are summarised in Table 3.

Table 3

Primer/Probe sequences

Designation	**Primer Sequence**	**SEQ ID NO**
Anchor1	TTTTTTTTTTTTTTTTTACGCCGGCGCTTAA GTACGCTCGGGCT	14

Anchor1 - R1	TCGGGCTCGCATGAATTCG	15
Anchor1 - R2	ATGAATTCGCGGCCGCAT	16
Anchor1 - R3	TCGGGCTCGCATGAATTCGCG	17
Anchor1 - R4	CTCGCATGAATTCGCGGCCGC	18
F1	ATHTGYCANCCNGCNGG	19
F2	ATHTGYCANCCNGCNGGNGT	20
F3	CANCCNGCNGGNGTNAA	21
F4	CCNTGYTGYCCNGGNYT	22
F5	TNAARTGYATHGGNGG	23
F6	GGNGTNAAYAAYAARGTNTG	24
F7	AARATHTGYACICCIGCIGGIGTIAA	25
F8	CCIGCIGGIGTIAARTGYCCIGCIGC	26
F9	TGYCCIGCIGCIYTICCITGYTGYCC	27
F10	TGYATHGGIGGIGTIAAYAAYAARGT	28
F11	TAAATGTCCCGCGGCTCTTCC	29
F12	CGGCTCTTCCTTGCTGCCCCG	30
F13	TGCTGCCCCGGACTTCGCTGC	31
Anchor3	HO-GTTTAATTACCCAAGTTTGAGNNNNNN - NH_2	32
Anchor3 - attach	PO_4 - CTCAAACTTGGTAATTAAACC - NH_2	33
Anchor3 - F1	GGTTTAATTACCCAAGTT	34
Anchor3 - F2	TAATTACCCAAGTTTGAG	35
Anchor3 - F3	GGTTTAATTACCCAAGTTTGAG	36
R1	CAIACYTTRTTRTTIACICCICC	37
R2	ATGCAGCGAAGTCCGGGGCAG	38
R3	GGGGCAGCAAGGAAGAGCCGC	39
R4	AAGAGCCGCGGGACATTTAAC	40
Probe F	AGTTAAATGTCCCGCGGCTCTTCCTTGCTG CCCCGGACTTCGCTGCATC	41
Probe R	GATGCAGCGAAGTCCGGG	42

where "F" designates a forward primer and "R" designates a reverse primer.

I. RACE PCR

First strand cDNA synthesis

The ClonTech Advantage RT-for-PCR kit was used for this, in accordance with the manufacturers' specifications.

20 pmol oligo(d)T primer 'Anchor1' was annealed to 1 µg total RNA in a total volume of 13.5 µl by heating to 70°C for 2 minutes and rapidly quenching on ice.

The following reaction components were added:

4 µl 5 x reaction buffer

0.5 µl RNase inhibitor

1 µl MMLV reverse transcriptase

1 µl 10mM dNTP

The reaction was incubated at 42°C for 1 hour, and the reaction stopped by denaturing the enzyme at 95°C for 5 minutes. 80 µl RNase free water was added and cDNA stored at -80°C.

3' RACE PCR

5 µl of reaction mix from the first strand cDNA synthesis reaction was used as a template with various primer set combinations to amplify the 3' end of the peptide coding cDNA. The primers (see Table 3) used were degenerate, and designed on the known amino acid sequence of the N-terminal end of the mature peptide to allow for selective amplification.

PCR reactions were performed using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech). These contain all necessary components for a PCR reaction as a bead in a 0.5 ml tube. The following components were added:

cDNA template	5 µl reaction mix from cDNA synthesis step

Forward primer	25 pmol
Reverse primer	25 pmol
Sterile Water	to a final volume of 25 µl .

PCR cycle conditions

95 °C 1 min

↓

95 °C 1 min

↓

64 °C* 1 min — 30 cycles

↓

72 °C 1 min

↓

72 °C 10 min

*Annealing temperature varied depending on primer set

PCR products were visualised by agarose gel electrophoresis on a 1% agarose gel in TBE buffer. Discrete PCR products were cloned into pCR2.1 TOPO using the Invitrogen TOPO TA cloning kit according to the manufacturers' specification.

Each ligation contained

1 µl	PCR product
1 µl	pCR2.1 TOPO vector
3 µl	Sterile Water

and was incubated at room temperature for 5 minutes. 2 µl of each ligation mix was transformed into TOP10 competent cells by heat shock at 42°C for 30 seconds followed by

incubation on ice for 2 minutes. Transformed cells were allowed to express beta-lactamase by incubation at 37°C in SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) for 1 hour with shaking at 225 rpm.

Cells were plated on Luria-Bertani Agar plates (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 15g/L agar, 0.006% X-gal, 0.15mM IPTG) containing 50µg/ml kanamycin for plasmid transformant selection and to enable identification of those containing recombinant TOPO TA isolates.

Discrete white colonies were selected from different PCR TOPO TA reactions, grown overnight in 5 ml Luria-Bertani (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, in water, pH 7.0) containing 50µg/ml kanamycin.

Plasmid DNA was extracted from the cultures using the Wizard DNA purification kit (Promega), following manufacturers' specifications. DNA was eluted in 50µl sterile water. Plasmid DNA was digested with *EcoRI* to confirm the presence and size of inserts.

3 µl	Plasmid DNA
1 µl	*EcoRI* (Kramel Biotech)
1 µl	10 x Restriction Buffer 6 (Kramel Biotech)
5 µl	Sterile water

Digests were incubated at 37°C for 2 hours and the presence or absence and size of inserts determined by agarose gel electrophoresis.

Based on these analyses, recombinant plasmids were selected for sequencing on a Perkin Elmer ABI 377XL DNA sequencer with the ABI Prism dye terminator cycle sequencing ready reaction kit, according to the manufacturers' protocol.

4 pmol	primer M13 Univ or M13 R
5 µl	DNA

Sterile water to 12 μl

The coding sequence of the peptide of SEQ ID NO 1 was identifiable by translation of the nucleotide sequence into amino acid sequence in all possible reading frames and comparison of this sequence to the known amino acid sequence of the peptide. This analysis used the DNA Star sequence analysis software (SeqMan, EditSeq, Macaw, VectorNTI).

5' RACE PCR

An anchor-ligation approach (Troutt, A.B., et al., *Proc. Natl. Acad. Sci. USA*. **89**, 9823-9825) was used to obtain the nucleotide sequence of the 5' half of the 524445 gene. This entailed attachment of a specific anchor primer to the 5' end of the first strand cDNAs. Use of this sequence together with mRNA specific for the peptide of SEQ ID NO 1 complementary 3' primers allowed for selective amplification of the 5' end of the corresponding coding cDNA.

i. Primer annealing

Complementary oligonucleotides Anchor3 and Anchor3-attachment were annealed to each other in equimolar ratio's at three different final concentrations (1nM, 100nM, 10mM).

Oligonucleotide mixtures were heated to 95°C and cooled slowly to 45°C for annealing.

ii. Ligation of annealed primer to cDNA

The attachment primer is complementary to the anchor primer, but contains a 3' extension of 5 additional fully degenerate bases i.e. synthesised with A, G, C and T at each position. This degenerate 'tail' allows individual attachment primers to anneal to the 3' terminus of any cDNA molecule. An amido group at the 3' end of the primer blocks DNA synthesis. A phosphate group at the 5' end of the anchor primer allows ligation of this to the 3' end of the cDNA molecules to provide a specific recognition sequence for PCR amplification.

Reactions for ligation of annealed anchor primers to first strand cDNA preparations contained:

5 μl	reaction mix from first strand cDNA synthesis
30 mM	Tris HCl (pH 8)
10 mM	$MgCl_2$
10 mM	Dithiothreitol
0.5 mM	ATP
1 μl	T4 DNA ligase (4 U/μl) (Kramel Biotech)
1 μl	Water
1 μl	Annealed anchor primers (final concentrations of 100mM, 10nM, 1mM)

Reactions were cycled overnight as follows:

25 °C 5 min

↕ Slow Ramp Rate

4 °C 5 min

Reactions were pooled, incubated at 95°C for 5 minutes and snap frozen in liquid nitrogen. After thawing on ice, excess primers were removed by purification through a Wizard PCR clean-up column (Promega) using the manufacturers' specifications. cDNAs were eluted in 40 μl water.

iii. RACE PCRs

PCR reactions were set up using the anchor-linked cDNA as a template, specific forward primers based on this anchor sequence, and specific primers based on the gene sequence of the peptide of SEQ ID NO 1 identified previously by 3'RACE.

PCR reactions were performed using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech) as used previously for 3' RACE. Components added to the PCR beads were:

1 µl	cDNA template with Anchor3 annealed to 3' end of first strand cDNA
20 pmol	Forward primer
20 pmol	Reverse primer
	Sterile water to total volume of 25 µl.

PCR cycle conditions were:

95 °C 1 min

↓

95 °C 1 min

↓

58 °C* 1 min — 30 cycles

↓

72 °C 1 min

↓

72 °C 10 min

*Annealing temperature varied depending on primer set

PCR products were visualised by agarose gel electrophoresis and TOPO cloned as described above. Plasmid DNA was extracted from clones carrying candidate recombinant plasmids by Wizard miniprep, *EcoRI* digested and sequenced, as performed previously for 3' RACE clones (described above).

II. cDNA Library

Library Construction

A cDNA library of the fungus *Paecilomyces farinosus* was constructed using the lambda-ZAP cDNA synthesis and ZAP-cDNA Gigapack III Gold Cloning kit from Stratagene, according to the manufacturers' specifications unless stated.

Double stranded cDNA was synthesised using 5 µg the mRNA from the peptide of SEQ ID NO 1 (see above) as a template. This involved first and second strand cDNA synthesis, blunting of cDNA termini, ligation of adapters, and digestion with specific restriction enzymes to produce appropriate 'sticky ends' for directional cloning.

A Sephacryl S-400 HR MicroSpin column (Amersham Pharmacia Biotech) was used to remove excess adapters rather than the size fractionation step suggested in the kit. The gel filtration medium provided in the kit (sepharose CL-2B) separates molecules on the basis of size with a cut-off of 400bp. As the mature insecticidal peptide is only 33 amino acids long, it is highly likely that the gene may be smaller than 400bp and would have been selected against using the sepharose filtration medium.

cDNAs were ligated into the Uni-ZAP XR vector and packaged into phage. The library titre was 2.5 million clones, with an average insert size of 700bp ranging from 150bp to 2 Kb.

Library screening

A total of 500,000 plaques were plated on Luria-Bertani Agar plates according to the cDNA library manufacturers' specification. Duplicate lifts of the plaques were made onto nitrocellulose membrane (Hybond-N, Amersham Pharmacia Biotech).

The membranes were prehybridised in Denhardts hybridisation solution (5x SSPE, 5x Denhardt's Reagent [50x Denhardt's Reagent: 5g Ficoll, 5g polyvinylpyrrolidone, 5g bovine serum albumin, sterile water to 500ml], 0.5% SDS, sterile water to 1L) containing 200µlsalmon sperm DNA (10mg/ml) which had been denatured by boiling for 10 minutes, for 2 hours at 65°C. A radioactive probe was prepared by end labelling an oligonucleotide specific for the coding sequence of a peptide of SEQ ID NO 1:

25 ng	Oligonucleotide (445-F11)
1 µl	Polynucleotide Kinase Buffer (Kramel Biotech)
1.5 µl	T4 Polynucleotide Kinase (Kramel Biotech)
5 µl	gamma 32P dATP
	Sterile Water to 10 µl

The probe was incubated at 37°C for 5 hours. The probe was added to 50ml Denhardts hybridisation solution and hybridised overnight at 65°C.

Membranes were washed in a 0.1x SSC, 0.1% SDS solution for 4 x 15 minutes to remove unbound probe. Exposure to x-ray film identified positive plaques containing 524445 coding sequence.

Positive plaques were cored from the original agar plates into 1ml SM buffer (5.8g NaCl, 2g $MgSO_4.7H_2O$, 50ml 1M Tris-Hcl pH 7.5, 5ml 2% gelatin, sterile water to 1L) containing 20ml chloroform and vortexed. The phage DNA was allowed to enter the phage buffer by storage at 4°C overnight. Samples of phage were then diluted and re-plated to obtain approximately 200 plaques per plate. The plaque lift and hybridisation procedure above was repeated to identify positives.

This process was followed for three rounds of screening until the plaques were pure. Self excision of 12 candidate positive plaques into colonies was performed as per Stratagene's specifications with the cDNA library kit.

Candidate colonies were grown overnight in 5ml Luria-Bertani medium containing 100µg /ml ampicillin, plasmid DNA extracted using Promega's Wizard miniprep kit, and the inserts sequenced using M13 Universal and M13 Reverse primers (see above for details of all).

Nucleotide Sequence

The nucleotide sequence of the peptide of SEQ ID NO 1 is shown in Figure 2. The putative translation initiation codon and stop codon are shown in italics. The sequence which codes for the mature peptide is underlined.

The sequence in Figure 2 indicates that there is approximately a 110 nucleotide 5' non-coding sequence and a 160 nucleotide 3' non-coding sequence. There seems to be a 17 amino acid signal peptide 5' of the mature coding sequence. Potential signal sequence cleavage sites were predicted based on the method of von Heijne, G. (1986). *Nucleic Acids Research.* 14, 4683. The potential cleavage site is indicated by a downward pointing arrow. It is probable that a secondary processing event removes the signal peptide from the mature peptide, e.g. by signal peptidase cleavage.

<u>Example 7</u>

<u>Expression of peptide in corn</u>

European Corn Borer (*Ostrinia nubilalis*) and therefore corn, *Zea mays,* which has been transformed so as to express this peptide would be expected to be protected against this pest.

Suitable constructs for expression in corn can be summarised as follows:

Construct	*Promoter*	*Signal Peptide*	*Gene*	*Terminator*
1	Maize Ubi	SEQ ID NO 10	SEQ ID NO 9+	nos
2	Maize Ubi	SEQ ID NO 3	SEQ ID NO 9+	nos
3	Maize Ubi	SEQ ID NO 7 *	SEQ ID NO 9+	nos

* This signal peptide contains an internal *NcoI* site which can be mutated (for example CCATGG → CTATGG) to destroy it if *NcoI* is required for cloning.

+ The natural coding sequence can be modified in accordance with the degeneracy of the genetic code, and in particular for the purpose of codon optimisation in corn.

The signal peptide can be fused to the mature gene for example using an overlapping PCR approach as illustrated in Figure 4. The fusion is suitably designed with restriction sites to allow cloning into monocot vectors. For example, it may comprise the following:

5' *NcoI - KpnI* ------- Signal ------- Gene ------- *SacI - HindIII* 3'

The full length signal-gene fusion can be ligated between the maize ubiquitin promoter and nos terminator into a backbone vector containing PAT selection (phosphinothricin - basta herbicide resistance).

These constructs can be used to transform corn cells which can then be grown into callus as is well known in the art. The transformed callus can be subjected to a corn callus transient assay and/or an *in vivo* bioassay to confirm expression and activity of the peptide.

Example 8

Expression of Peptide in Cotton

The peptide of the invention has good activity against the Beet Armyworm (*Spodoptera exigua*) which is a major cotton pest. Thus cotton *Gossypium hirsutum*, which has been transformed to express this peptide would be protected against this pest.

Suitable constructs for use in the transformation in this case can be summarised as:

Construct	*Promoter*	*Signal Peptide*	*Gene*	*Terminator*
4	RolDFd	SEQ ID NO 13	SEQ ID NO 9	potato protease inhibitor II
5	RolDFd	SEQ ID NO 3	SEQ ID NO 9	potato protease inhibitor II
6	RolDFd	SEQ ID NO 7	SEQ ID NO 9	potato protease inhibitor II

The signal peptide can be fused to the mature gene using an overlapping PCR approach as in Example 7. In this case, the fusion is suitably designed with restriction sites to allow cloning into dicot vectors. The full length signal-gene fusion can be ligated into a housekeeping vector between the RolDFd promoter and potato protease inhibitor II terminator. The entire cassette could then be cut out using restriction enzymes and ligated into an appropriate binary vector.

Constructs can then be tested using conventional methods.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

Example 9

Insecticidal activity of the protein combination

Previously prepared European Corn Borer (ECB) artificial diet was dispensed in small quantities into tubes and held in a warm water bath at 70°C. To each tube containing 975μl of diet, 75μl of the appropriate test sample was added. The test samples comprised a mixture of the cry1Ia1 protein (SEQ ID No. 44) and the protein depicted as SEQ ID No. 1 (the R524445 protein). The "incorporated diet" was mixed well and 180μl aliquots were then pipetted out onto Falcon™ 1006 petri dishes, giving five replicates for each sample. The dishes were infested 1 - 5 hours after the diet is dispensed with five 1st instar larvae per dish/rep and then lidded. The test was held in the dark at 27°C and 70 - 80% RH and the insects were assessed five days after treatment for mortality. The results are shown in the Table 4 below:

Table 4

(Results shown as number of insects living)/(number of insects within the dishes)						
cry1Ia1 Conc. (PPM)	R524445 Protein concentration (PPM)					
	0.00	4.91	7.60	11.75	18.16	22.4
0.00	15/15	13/15	10/14	8/15	6/15	5/15
2.48	10/11	1/14	0/15	0/15	0/15	-
3.65	7/11	1/15	0/15	0/15	0/15	-
5.36	3/13	0/15	0/15	0/15	0/15	-
7.87	1/15	0/15	1/14	0/15	0/15	-
11.57	2/13	-	-	-	-	-

CLAIMS

1. An insecticidal synergistic combination comprising a first protein depicted as SEQ ID No 43 and at least one further protein.

2. A combination according to claim 1 wherein the said first protein comprises the sequence depicted as SEQ ID No 1.

3. A combination according to claim 1 or claim 2 wherein the said further protein is an insecticidal CRY protein.

4. A combination according to claim 3 wherein the said further protein is selected from the group consisting of SEQ ID Nos. 44 to 49.

5. A polynucleotide which comprises a region encoding the said first and further protein according to any one of claims 1 to 4.

6. A method of providing a plant or plant part with a combination according to any one of claims 1 to 4 comprising:
(a) inserting into a plant cell a polynucleotide according to claim 5; and
(b) regenerating a plant from said cell; and
(c) selecting the plant or plant part having the said combination.

7. A method of providing a plant or plant part with a combination according to any one of claims 1 to 4 comprising:
(a) inserting into a plant cell which is capable of producing a further protein, a polynucleotide which provides for the production of a first protein depicted as SEQ ID No 43; and
(b) regenerating a plant from the said cell; and
(c) selecting the plant or plant part having the said combination.
or,

(a) inserting into a plant cell which is capable of producing a first protein depicted as SEQ ID No 43, a polynucleotide which provides for a further protein; and
(b) regenerating a plant from the said cell; and
(c) selecting the plant or plant part having the said combination.

8. A method of providing a plant with a combination according to any one of claims 1 to 4 comprising crossing a first plant which is capable of producing a first protein depicted as SEQ ID No 43 with a second plant which is capable of producing a further protein and selecting the resultant plant which is capable of producing the said combination

9. A method according to claim 7 or 8 wherein the said first protein has the sequence depicted as SEQ ID No 1 and the said further protein is an insecticidal CRY protein.

10. A plant obtained by the method of any one of claims 6 to 9.

11. A plant cell which comprises an insecticidal synergistic combination according to any one of claims 1 to 4.

12. A plant comprising cells according to claim 11.

13. A method of controlling insects comprising providing at a locus where the said insects feed, a combination according to any one of claims 1 to 4.

14. A method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising:
(a) providing a population of variants of said polynucleotide and further polynucleotides which encode further proteins, at least one of which is in cell free form; and
(b) shuffling said variants and further polynucleotides to form recombinant polynucleotides; and

(c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and

(d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least a polynucleotide encoding the protein depicted as SEQ ID No. 43.

15. A method according to claim 14 characterised in that the said population of variants in part (a) contains at least a polynucleotide encoding the protein depicted as SEQ ID No.43 and at least one of the said further polynucleotides encodes a CRY protein.

16. A method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising:

(a) providing a population of variants of said polynucleotide and further polynucleotides which encode further proteins, at least one of which is in cell free form; and

(b) shuffling said variants and further polynucleotides to form recombinant polynucleotides; and

(c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and

(d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least a polynucleotide according to claim 5.

17. A method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising:

(a) providing a population of variants of said polynucleotide, at least one of which is in cell free form; and

(b) shuffling said variants to form recombinant polynucleotides; and

(c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and

(d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least a polynucleotide encoding the protein depicted as SEQ ID No. 43.

18. A method according to claim 17 characterised in that the said population of variants in part (a) contains at least a polynucleotide encoding the protein depicted as SEQ ID No. 1.

19. A polynucleotide obtainable by the method according to any one of claims 14 to 18.

20. A polynucleotide obtained by the method according to any one of claims 14 to 18.

21. A protein encoded by a polynucleotide according to claim 19 or claim 20.

22. Use of an evolved polynucleotide according to claim 19 or claim 20 in the production of plants or plant parts which are resistant to infestation by insects.

23. Use of an evolved polynucleotide according to claim 19 or claim 20 in the production of a protein which is capable of producing a synergistic combination with a further protein.

24. A polynucleotide encoding a fusion protein comprising a first protein depicted as SEQ ID No 43 and at least one further protein.

25. A polynucleotide according to claim 24 wherein the said first protein has the sequence depicted as SEQ ID No. 1 and the said further protein is a CRY protein.

26. A polynucleotide according to claim 25 wherein the said further protein is selected from the group consisting of SEQ ID Nos. 44 to 49.

27. A polynucleotide according to any one of claims 24 to 26 which further comprises a sequence encoding a region which separates the said first and further proteins within the said fusion protein.

28. A fusion protein encoded by a polynucleotide according to any one of claims 24 to 27.

29. A method of evolving a polynucleotide which encodes a protein having insecticidal properties comprising:
(a) providing a population of variants of said polynucleotide, at least one of which is in cell free form; and
(b) shuffling said variants to form recombinant polynucleotides; and
(c) selecting or screening for recombinant polynucleotides which have evolved towards the said insecticidal properties; and
(d) repeating steps (b) and (c) with the recombinant polynucleotides according to step (c) until an evolved polynucleotide which encodes a protein having insecticidal properties has been acquired characterised in that the said population of variants in part (a) contains at least a polynucleotide according to any one of claims 24 to 27.

30. A plant comprising a polynucleotide according to claim 19, 20, 24 to 27 and 29.

Figure 1

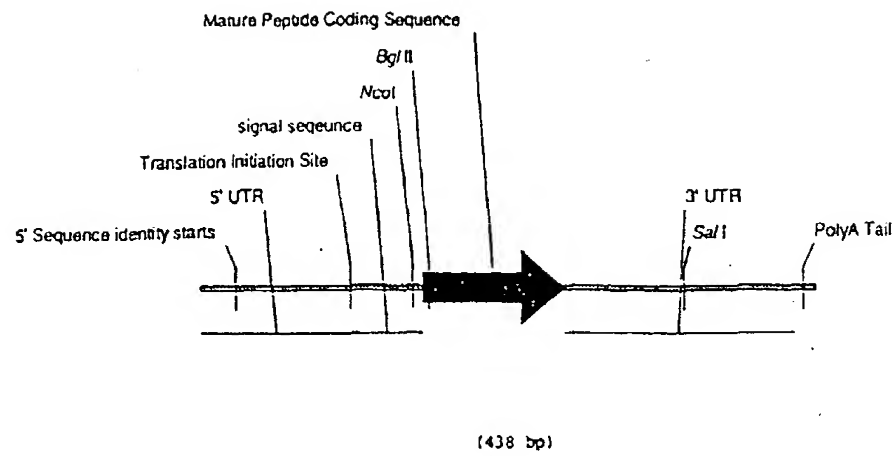


Figure 2

(SEQ ID NO 2)

```
  1  ATTACCCAAG TTTGAGGGCA TTCAATTTCA CACAGTCTCA CGCTTTCGAC

 51  GCATCTACTT CTTCGTCTCA CGCCATATAT CCTCCCAAAA TCACACCTCT

 +2             Met GlnIleSer AlaValIle ValAlaLeuPhe AlaSerAl
101  TCCTTCACCA TGCAAATCTC CGCCGTCATT GTCGCACTCT TCGCCAGCGC

 +2 aAlaMetAla GlyLysIleCys ThrProAla GlyValLys CysProAlaA
151  CGCCATGGCC GGCAAGATCT GCACTCCTGC TGGAGTTAAA TGTCCCGCGG

 +2  laLeuProCys CysProGly LeuArgCysIle GlyGlyVal AsnAsnLys
201  CTCTTCCTTG CTGCCCCGGA CTTCGCTGCA TCGGCGGCGT CAACAACAAG

 +2  ValCysArg***
251  GTTTGCCGGT AATTCTAGTG TCGCAACTTT TGAGCGTGGG ATAAGTATGC

301  TTCGTTCGTT GTATGGAGTT CTCCTCCGGA GTTTAAGCTC GGCCGGTCGA

351  CAGCGGGTCT GCTATACTTG ATCTTACAGC GATACTATTG ATAGAAATGC

401  ACATCTTCAT TCATGCGTCA TGAAAAAAAA AAAAAAAA
```

Signal peptide sequence indicated in bold type

Mature peptide sequence underlined

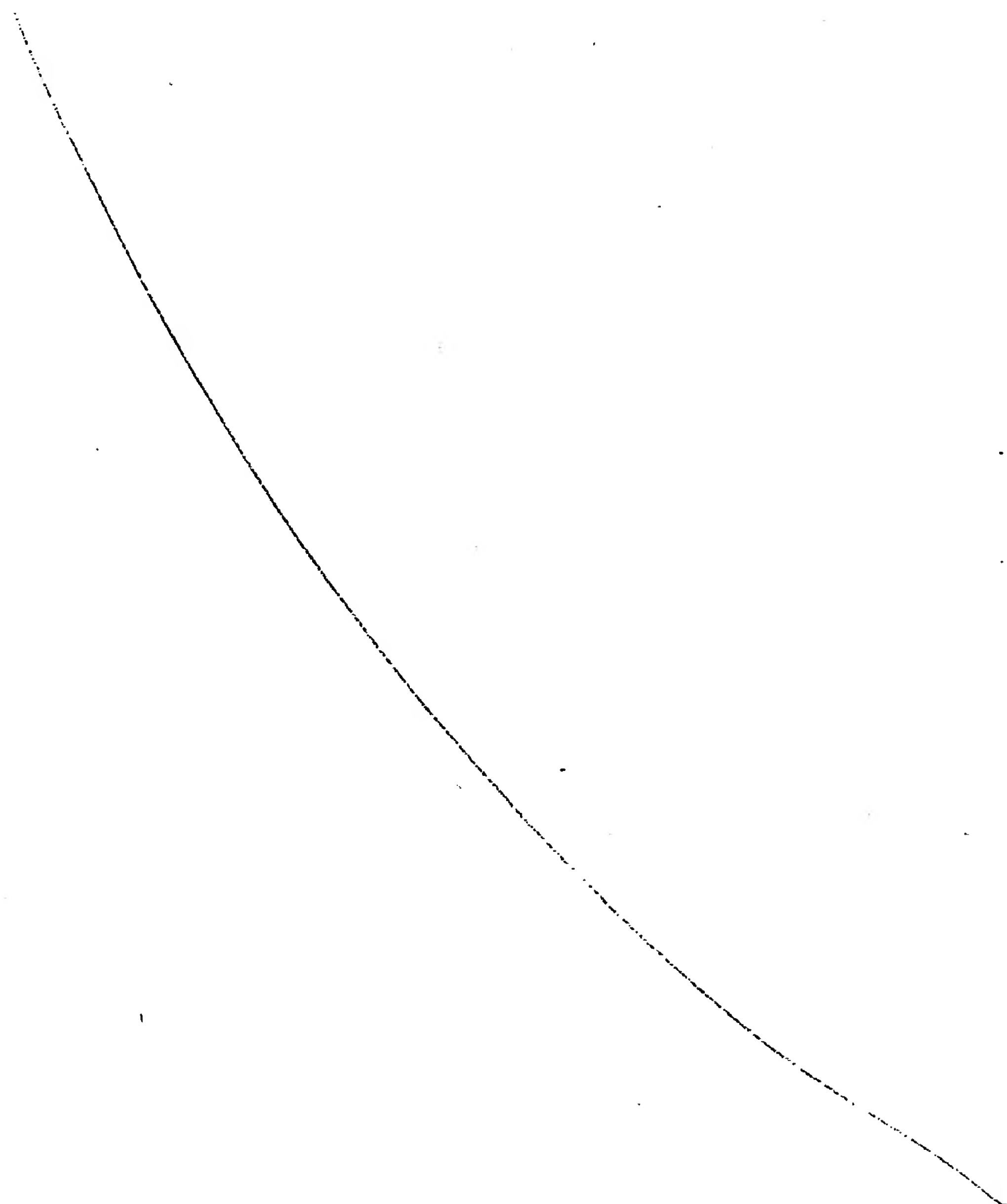


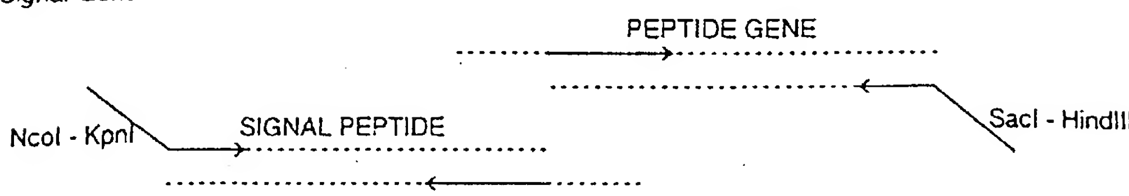
Figure 3

(SEQ ID NO 9)

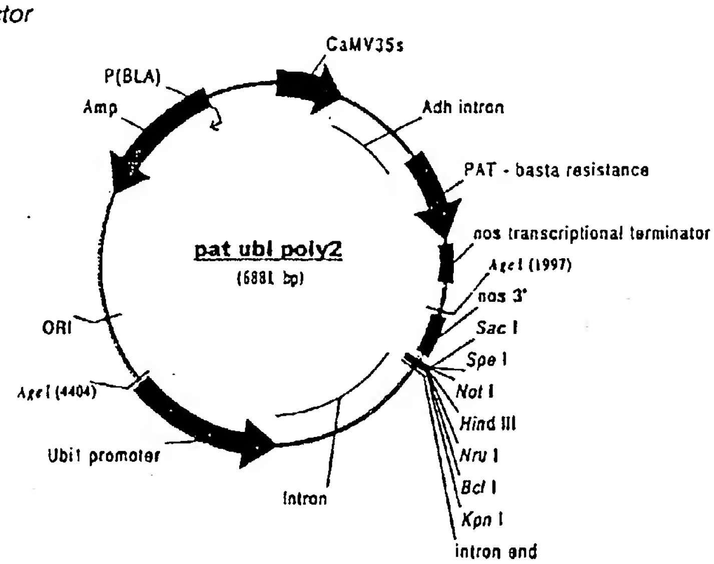
GGCAAGATCT GCACTCCTGC TGGAGTTAAA TGTCCCGCGG CTCTTCCTTG
CTGCCCCGGA CTTCGCTGCA TCGGCGGCGT CAACAACAAG GTTTGCCGGT AA

Figure 4

A
Signal-Gene Fusion



B
Backbone Vector



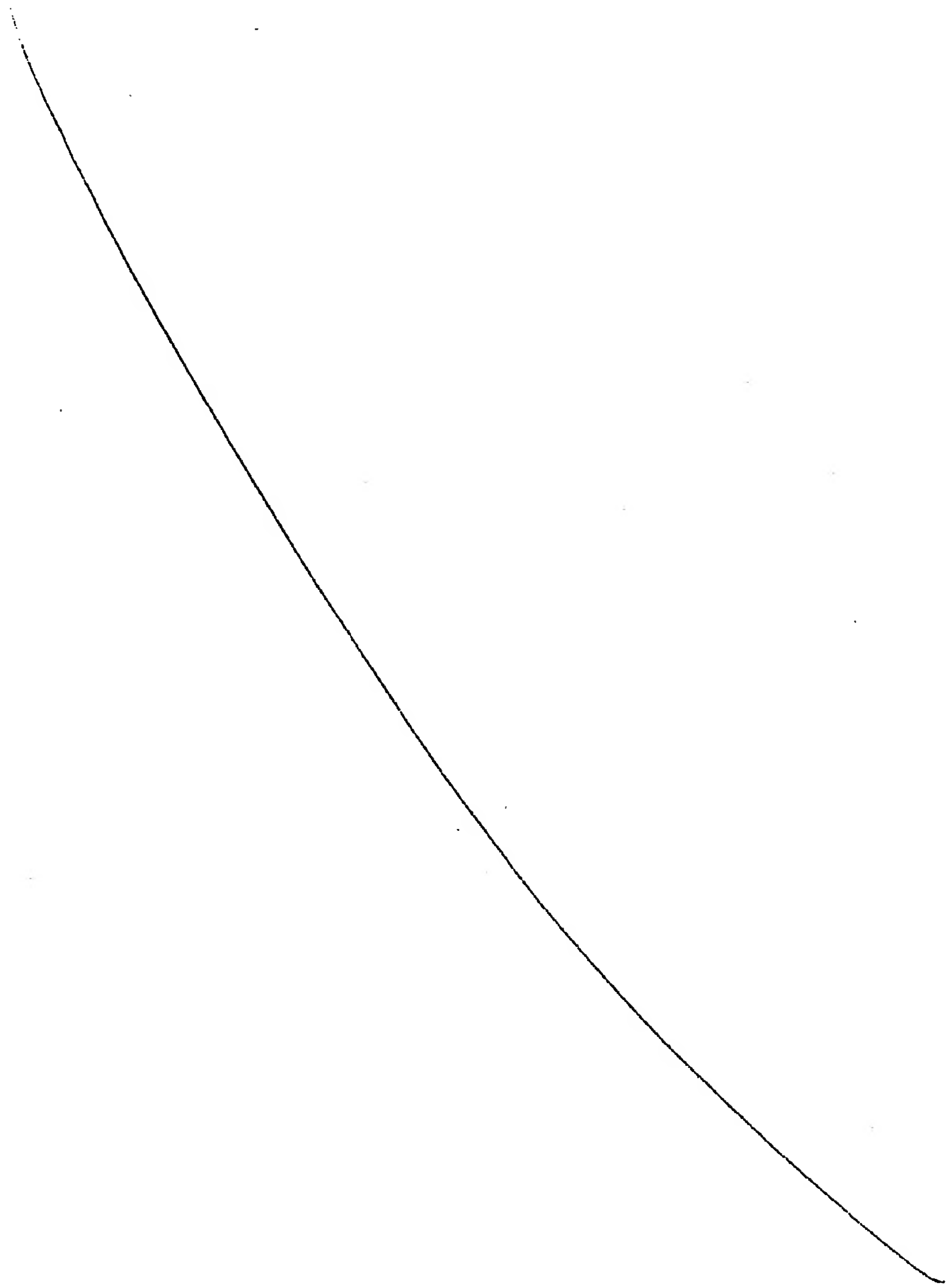


FIGURE 5

SEQ ID No. 43

$-AA_1-AA_2-AA_3-Cys_4-AA_5-AA_6-AA_7-AA_8-AA_9-AA_{10}-Cys_{11}-AA_{12}-AA_{13}-AA_{14}-AA_{15}-AA_{16}-Cys_{17}-Cys_{18}-AA_{19}-AA_{20}-AA_{21}-AA_{22}-Cys_{23}-AA_{24}-AA_{25}-AA_{26}-AA_{27}-AA_{28}-AA_{29}-AA_{30}-AA_{31}-Cys_{32}-AA_{33}$ (SEQ ID No 43) or a fragment thereof, with the *proviso* that the amino acids depicted at positions AA1-3, 5-10, 12-16, 19-22, 24-31 and 33 refer to any amino acid other than cysteine.

FIGURE 6

SEQ ID No. 44 cry1Ia1 (Embl. Accession No. X62821)

MKLKNQDKHQSFSSNAKVDKISTDSLKNETDIELQNINHEDCLKMSEYENVEPFV
SASTIQTGIGIAGKILGTLGVPFAGQVASLYSFILGELWPKGKNQWEIFMEHVEEII
NQKISTYARNKALTDLKGLGDALAVYHDSLESWVGNRNNTRARSVVKSQYIAL
ELMFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLLRDASIFGKEWGLSSSEISTFY
NRQVERAGDYSYHCVKWYSTGLNNLRGTNAESWVRYNQFRRDMTLMVLDLV
ALFPSYDTQMYPIKTTAQLTREVYTDAIGTVHPHPSFTSTTWYNNNAPSFSAIEAA
VVRNPHLLDFLEQVTIYSLLSRWSNTQYMNMWGGHKLEFRTIGGTLNISTQGST
NTSINPVTLPFTSRDVYRTESLAGLNLFLTQPVNVPRVDFHWKFVTHPIASDNFY
YPGYAGIGTQLQDSENELPPEATGQPNYESYSHRLSHIGLISASHVKALVYSWTH
RSADRTNTIEPNSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRVN
INPPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSATMNRGEDLDYKTFXTV
GFTTPFSLLDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAEYDFEKAQEKVT
ALFTSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEKRELFEIVKYAKQLHIER
NM

FIGURE 7

SEQ ID No. 45 cry1Ia2 (Embl. Accession No. M98544)

MKLKNQDKHQSFSSNAKVDKISTDSLKNETDIELQNINHEDCLKMSEYENVEPFV
SASTIQTGIGIAGKILGTLGVPFAGQVASLYSFILGELWPKGKNQWEIFMEHVEEII
NQKISTYARNKALTDLKGLGDALAVYHDSLESWVGNRNNTRARSVVKSQYIAL
ELMFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLLRDASIFGKEWGLSSSEISTFY
NRQVERAGDYSDHCVKWYSTGLNNLRGTNAESWVRYNQFRRDMTLMVLDLV
ALFPSYDTQMYPIKTTAQLTREVYTDAIGTVHPHPSFTSTTWYNNNAPSFSAIEAA
VVRNPHLLDFLEQVTIYSLLSRWSNTQYMNMWGGHKLEFRTIGGTLNISTQGST
NTSINPVTLPFTSRDVYRTESLAGLNLFLTQPVNGVPRVDFHWKFVTHPIASDNF
YYPGYAGIGTQLQDSENELPPEATGQPNYESYSHRLSHIGLISASHVKALVYSWT
HRSADRTNTIEPNSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRV
NINPPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSATMNRGEDLDYKTFRT
VGFTTPFSFLDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAEYDFEKAQEKV
TALFTSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEKRELFEIVKYAKQLHIE
RNM

FIGURE 8

SEQ ID No. 46 cry1Ia3 (Embl. Accession No. L36338)

MKLKNQDKHQSFSSNAKVDKISTDSLKNETDIELQNINHEDCLKMSEYENVEPFV
SASTIQTGIGIAGKILGTLGVPFAGQVASLYSFILGELWPKGKNQWEIFMEHVEEII
NQKISTYARNKALTDLKGLGDALAVYHDSLESWVGNRNNTRARSVVKSQYIAL
ELMFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLLRDASIFGKEWGLSSSEISTFY
NRQVERAGDYSYHCVKWYSTGLNNLRGTNAESWVRYNQFRRDMTLMVLDLV
ALFPSYDTQMYPIKTTAQLTREVYTDAIGTVHPHPSFTSTTWYNNNAPSFSAIEAA
VVRNPHLLDFLEQVTIYSLLSRWSNTQYMNMWGGHKLEFRTIGGTLNISTQGST
NTSINPVTLPFTSRDVYRTESLAGLNLFLTQPVNGVPRVDFHWKFVTHPIASDNF
YYPGYAGIGTQLQDSENELPPEATGQPNYESYSHRLSHIGLISASHVKALVYSWT
HRSADRTNTIEPNSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRV
NINPPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSATMNRGEDLDYKTFRT
VGFTTPFSFLDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAEYDFEKAQEKV
TALFTSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEKRELFEIVKYANELHIE
RNM

FIGURE 9

SEQ ID No. 47 cry1Ia4 (Embl. Accession No. L49391)

MKLKNQDKHQSFSSNAKVDKISTDSLKNETDIELQNINHEDCLKMSEYENVEPFV
SASTIQTGIGIAGKILGTLGVPFAGQVASLYSFILGELWPKGKNQWEIFMEHVEEII
NQKISTYARNKALTDLKGLGDALAVYHDSLESWVGNRNNTRARSVVKSQYIAL
ELMFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLLRDASIFGKEWGLSSSEISTFY
NRQVERAGDYSDHCVKWYSTGLNNLRGTNAESWVRYNQFRRDMTLMVLDLV
ALFPSYDTQMYPIKTTAQLTREVYTDAIGTVHPHPSFTSTTWYNNNAPSFSAIEAA
VVRNPHLLDFLEQVTIYSLLSRWSNTQYMNMWGGHKLEFRTIGGTLNISTQGST
NTSINPVTLPFTSRDVYRTESLAGLNLFLTQPVNGVPRVDFHWKFVTHPIASDNF
YYPGYVGIGTQLQDSENELPPEATGQPNYESYSHRLSHIGLISASHVKALVYSWT
HRSADRTNTIEPNSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRV
NINPPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSATMNRGEDLDYKTFRT
VGFTTPFSFLDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAEYDFEKAQEKV
TALFTSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEKRELFEIVKYAKQLHIE
RNM

FIGURE 10

SEQ ID No. 48 cry1Ia5 (Embl. Accession No. Y08920)

MKLKNQDKHQSFSSNAKVDKISTDSLKNETDIELQNINHEDCLKMSEYENVEPFV
SASTIQTGIGIAGKILGTLGVPFAGQVASLYSFILGELWPKGKNQWEIFMEHVEEII
NQKISTYARNKALTDLKGLGDALAVYHDSLESWVGNRNNTRARSVVRSQYIAL
ELMFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLLRDASIFGKEWGLSSSEISTFY
NRQVERAGDYSDHCVKWYSTGLNNLRGTNAESWVRYNQFRRDMTLMVLDLV
ALFPSYDTQMYPIKTTAQLTREVYTDAIGTVHPHPSFTSTTWYNNNAPSFSAIEAA
VVRNPHLLDFLEQVTIYSLLSRWSNTQYMNMWGGHKLEFRTIGGTLNISTQGST
NTSINPVTLPFTSRDVYRTESLAGLNLFLTQPVNGVPRVDFHWKFVTHPIASDNF
YYPGYAGIGTQLQDSENELPPEATGQPNYESYSHRLSHIGLISASHVKALVYSWT
HRSADRTNTIEPNSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRV
NINPPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSATMNRGEDLDYKTFRT
VGFTTPFSFLDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAEYDFEKAQEKV
TALFTSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEKRELFEIVKYANELHIE
RNM

FIGURE 11

SEQ ID No. 50 cry1Ib1 (Embl. Accession No. U07642)

MKLKNPDKHQSLSSNAKVDKIATDSLKNETDIELKNMNNEDYLRMSEHESIDPF
VSASTIQTGIGIAGKILGTLGVPFAGQIASLYSFILGELWPKGKSQWEIFMEHVEEII
NQKILTYARNKALSDLRGLGDALAVYHESLESWVENRNNTRARSVVKNQYIALE
LMFVQKLPSFAVSGEEVPLLPIYAQAANLHLLLLRDASIFGKEWGLSASEISTFYN
RQVERTRDYSDHCIKWYNTGLNNLRGTNAKSWVRYNQFRKDMTLMVLDLVAL
FPSYDTLVYPIKTTSQLTREVYTDAIGTVHPNQAFASTTWYNNNAPSFSAIEAAVI
RSPHLLDFLEKVTIYSLLSRWSNTQYMNMWGGHRLESRPIGGALNTSTQGSTNTS
INPVTLQFTSRDVYRTESLAGLNLFLTQPVNGVPRVDFHWKFPTLPIASDNFYYL
GYAGVGTQLQDSENELPPETTGQPNYESYSHRLSHIGLISASHVKALVYSWTHRS
ADRTNTIEPNSITQIPLVKAFNLSSGAAVVRGPGFTGGDILRRTNTGTFGDIRVNIN
PPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSATMNRGEDLDYKTFRTIGFT
TPFSFSDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAEYDFEKAQEKVTALF
TSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEKRELFEIVKYAKQIHIERNM